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Characterization of bacteria isolated from maize roots: Emphasis on *Serratia* and infestation with corn rootworms (Chrysomelidae: *Diabrotica*)

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ABSTRACT

Larval corn rootworms (Coleoptera: Chrysomelidae) are soil-dwelling insect pests that damage maize (*Zea mays* L.) by consuming root tissues, thus lowering grain yield. Little is known about interactions between rootworms and soil bacteria, including potential impacts of maize rhizobacteria, such as entomopathogenic *Serratia* spp., on subterranean rootworm pests. We used selective growth medium (caprylate-thallous agar, CT) to quantify and isolate *Serratia* spp. from: (1) bulk soil, (2) roots of four field-grown maize genotypes, half of which were infested with rootworm eggs from a reared colony, and (3) non-diseased, larval rootworms from the same colony. Phenotypic testing and 16S rRNA gene sequencing were used to identify bacteria, including non-*Serratia* spp., that were successfully isolated on the CT solid medium. We also isolated and identified *Serratia* spp. associated with non-diseased and diseased *Diabrotica* adults. *Serratia* spp. associated with maize roots were more abundant than those associated with bulk soil, where they were undetectable with our methods except for *Serratia grimesii*. There was no impact of plant genotype on densities of bacteria isolated from maize roots. *S. grimesii* was frequently associated with maize roots, regardless of infestation with rootworm eggs. *Serratia marcescens* biotype A4, *Serratia plymuthica* and several other Enterobacteriaceae genera were also associated with maize roots. Infesting the soil with rootworm eggs enhance the occurrence of two strains of the *S. marcescens* biotype A1b, with comparable densities of these orange and pink strains within infested roots. However, both strains were associated with larval rootworms from the reared colony, which may indicate that rootworms were introducing these bacteria into infested maize roots. In addition, within larvae the orange strain was significantly more abundant than the pink strain, and was also associated with diseased rootworm adults. Our studies identified specific *Serratia* strains associated with diseased rootworms that may have potential as biological control agents, and additional *Serratia* biotypes associated with the maize rhizosphere that, based on the literature, may function as plant growth promoting agents via antagonistic action against plant-pathogenic fungi.

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1. Introduction

Corn rootworms (Coleoptera: Chrysomelidae) are devastating pests of maize (*Zea mays* L.). Although adult rootworms feed on developing ears and corn silks, the majority of plant damage is caused by subterranean larvae that consume maize root tissues, thus negatively impacting plant physiology and function (Riedell, 1990; Riedell and Reese, 1999). Ultimately, larval rootworm feeding reduces yield (Sutter et al., 1990; Spike and Tollefson, 1991), and can cause significant economic losses, which have been estimated at one billion U.S. dollars each year (Metcalf, 1986).

Interactions between plants, pests, and predators have been well studied for aboveground systems, but relatively little is known about subterranean ecological webs and plant-mediated protection against herbivores such as larval rootworms (Van der Putten et al., 2001). The role of rhizobacteria in plant protection against pathogenic bacteria and fungi (Elad et al., 1987; Press et al., 1997, 2001; Berg et al., 2000), plant-parasitic nematodes (Racke and Sikora, 1992; Siddiqui and Mahmood, 2001; Sturz and Kimpinski, 2004), and foliar-feeding arthropods (Zehnder et al., 2001) has received considerable attention, but information on interactions between rhizobacteria and rhizophagous insects is limited.

Maize roots have a diverse rhizobacterial community (Miller et al., 1989; Chelius and Triplett, 2001; Gomes et al., 2001; Brusetti et al., 2004) that includes several genera of Enterobacteriaceae, such as *Serratia* (Lalande et al., 1989; Lambert et al., 1987; McInroy and Kloepper, 1995). Microbes in this widespread genus are found within numerous habitats, such as soil, water, plants, and animals (Ashelford et al., 2002; Grimont and Grimont, 2006), and can have extremely different ecological functions, including plant pathogens (Bruton et al., 2003; Rascoe et al., 2003), biocontrol agents of plant pathogens (Ordentlich et al., 1988; Lottmann et al., 2000; Someya et al., 2001, 2005; Berg et al., 2006), plant growth promoters (Lalande et al., 1989; Hameeda et al., 2007), and insect symbionts (Iverson et al., 1984; Graber and Breznak, 2005; Moran et al., 2005; Lundgren et al., 2007). However, many *Serratia* strains are opportunistic pathogens of vertebrates and invertebrates (Grimont and Grimont, 2006), and are often associated with diseased and seemingly healthy insects (Lysenko, 1959; Steinhaus, 1959; Jackson et al., 2001).

Red-colored *Serratia* biotypes are associated with insects more often than non-pigmented biotypes (Grimont et al., 1979), while the former are rarely isolated from plants (Grimont et al., 1981). Although *Serratia* is highly toxic if it contaminates the hemocoel (Steinhaus, 1959; Grimont and Grimont, 2006), many strains also cause mortality when ingested (Steinhaus, 1959; Lysyk et al., 2002), or have sublethal effects such as reduced adult longevity and decreased oviposition and egg hatch (Sikorowski and Lawrence, 1998; Inglis and Lawrence, 2001; Sikorowski et al., 2001). *Serratia* can be transmitted vertically between one generation and the next, as well as horizontally between individuals within a generation (Sikorowski and Lawrence, 1998; Sikorowski et al., 2001). While records of mortality due to *Serratia* in natural insect populations are uncommon (Steinhaus, 1959), specific strains of *S. entomophila* and *S. proteamaculans* can kill grass grubs [*Costelytra zealandica* (White)], which are subterranean insect pests that damage plant roots (Jackson et al., 1993, 2001). Commercial pesticide formulations using *S. entomophila* have been developed for grass grub control (O'Callaghan and Gerard, 2005). However, there is little information about the extent and impact of sublethal *Serratia* infections in nature.

With regard to the maize rhizosphere, direct and indirect interactions between *Serratia*, host plants, and rootworms are poorly understood, and depending on bacterial strain identity, *Serratia* could be detrimental or beneficial to the plant. A strain of *Serratia marcescens* that causes cucurbit yellow vine disease (Rascoe et al., 2003) is vectored by the squash bug *Anasa tristis* (DeGeer) (Bruton et al., 2003). Rootworms can also vector plant pathogens (Palmer and Kommedahl, 1969; Gilbertson et al., 1986; Zehnder et al., 2001) and transfer rhizobacteria between plants (Snyder et al., 1998). Other rhizobacterial *Serratia* strains protect cucumber plants from bacterial wilt disease because the foliage of inoculated plants has lower densities of adult rootworms, which vector the disease (Zehnder et al., 2001).

Because some *Serratia* strains are insect pathogens (Steinhaus, 1959; Grimont and Grimont, 2006) that have been isolated from rootworms (Schalk et al., 1987; Tran and Marrone, 1988; Lance, 1992), and rootworms can acquire rhizobacteria when they feed on maize roots (Tran and Marrone, 1988), *Serratia* strains that colonize the maize rhizosphere may potentially function in defense against rootworms. In addition, it is possible that maize plants attacked by larval rootworms could actively defend themselves by selectively encouraging growth of entomopathogenic *Serratia* strains, because rhizobacterial communities are affected by root exudates (Richards, 1987; Walker et al., 2003; Brusetti et al., 2004; Bais et al., 2006), some of which are released in response to root damage (Denton et al., 1999; Grayston et al., 2001; Treonis et al., 2005; Ayres et al., 2007). Furthermore, the plant cultivar can influence root bacteria of some plant species (Miller et al., 1989; Germida and Siciliano, 2001), and thus maize genotype could potentially impact the resident rhizobacteria, and in turn affect host plant resistance to rootworms.

In order to elucidate interactions between maize roots, *Serratia* spp. rhizobacteria, and rootworms, and assess the pest biocontrol and/or plant growth promotion potential of associated microbes, the relevant bacteria must first be characterized. Our objectives were to: (1) quantify, isolate, and identify *Serratia* spp. associated with bulk field soil, maize roots, and larval western corn rootworms, *Diabrotica virgifera virgifera* LeConte, (2) determine if maize genotype and infesting soil with the eggs of *D. v. virgifera* impacts the presence or density of *Serratia* species and biotypes associated with bulk soil and maize roots, and (3) identify *Serratia* spp. associated with non-diseased and diseased adult rootworms. Our hypotheses were that: (1) a greater diversity of *Serratia* species and higher bacterial densities of these species would be associated with maize roots versus bulk soil, (2) plant genotype would influence the *Serratia* flora of maize roots, and (3) infestation with rootworm eggs would have a significant impact on the maize rhizobacterial flora.

2. Methods

2.1. Field sampling

Maize plants were grown at the Eastern South Dakota Soil and Water Research Farm near Brookings, SD, in a field previously managed under a four-year rotation of maize, soybeans, oats, and spring wheat to ensure that experimental plots were not contaminated by natural rootworm populations. In 2006, according to soil-testing recommendations 177 kg/ha starter (14-36-13) and 105 kg/ha urea (46-0-0) fertilizers were applied with a plot fertilizer spreader and incorporated into the top 10 cm of soil via field cultivation. On 5 May 2006 plots were sprayed with the pre-emergent herbicide Dual® II Magnum® (2.3 L/ha; Syngenta) and Roundup® (2.3 L/ha, Monsanto) for grass control, while on 5 June 2006 plots were sprayed with the post-emergent herbicide Callisto® (0.2 L/ha, Syngenta) for broadleaf weed control.

We isolated rhizobacteria from roots of four maize genotypes [CRW3(C6), NGSDCRW1(S2)C4, BS29-07-01, BS29-11-01], which have a range of agronomic characteristics and responses to rootworm infestation, including degree of root damage caused by larval western corn rootworm feeding and successful emergence of adult rootworms from the soil (Prischmann et al., 2007). In previous field experiments, CRW3(C6) had the least root damage of the four maize genotypes, whereas NGSDCRW1(S2)C4 had moderate root damage ratings and a large root system, and appeared tolerant to rootworm damage (Hibbard et al., 1999; Prischmann et al., 2007). BS29-07-01 and BS29-11-01 had the most root damage, although few adult rootworms emerged from BS29-07-01 plots (Prischmann et al., 2007).

A randomized complete block split plot design was used with four replicates. Within each replicate there were two whole plots, with infestation level as the whole-plot factor (non-infested versus infested with western corn rootworm eggs). Within each whole plot, there were four subplots, with maize genotype as the subplot-factor. Five plants of each maize genotype were planted consecutively within each single-row subplot. On 16 May 2006, seeds in experimental plots were hand-planted 5 cm deep with 23 cm within-row spacing. Seeds were not treated with any insecticides or fungicides. There were no formal buffer plants separating different maize genotypes within subplots, although two to four buffer plants were planted at the end of each whole plot. Rows with experimental plants were separated by buffer rows (DeKalb® 46-26), which were sown with seeds on 12 May 2006 at the recommended density of 63,010 seeds/ha. Row spacing was 0.75 m, and experimental single-row subplots were 1.15 m long.

Soil in infested whole plots was mechanically infested on 15 May 2006 with 1000 viable rootworm eggs per 30 cm of row suspended in a 0.15% agar solution (for details see Prischmann et al., 2007). Rootworm eggs were obtained from the primary diapausing colony maintained at the USDA-ARS North Central Agricultural Research Laboratory (NCARL) in Brookings, SD. Eggs were placed into the soil at a depth of 8–10 cm. Non-infested whole plots received the 0.15% agar solution without rootworm eggs.

On 20 July 2006, one of the three central plants from each set of maize genotypes within each subplot was processed. Therefore, 16 non-infested maize plants and 16 infested maize plants were sampled. Plant shoots were clipped off, roots dug using potato forks, loose soil shaken off, and roots placed in labeled plastic bags and transported to a refrigerator (4 °C). Soil samples were taken from non-infested soil ($n = 4$) and infested soil ($n = 4$) within experimental subplots where no plants were growing. Soil samples were taken approximately 0.15 m deep, and soil transferred to labeled plastic bags using a shovel.

In order to estimate rootworm development, on 16 May 2006 we placed the soil probe of a biophenometer (Model: BIO-51-TP03C; Omnidata® datapod, Logan, UT) 8.9–10.2 cm into the soil and monitored growing degree days with an upper threshold of 35 °C and a lower threshold of 11 °C. To confirm that rootworms successfully developed within infested treatments, on 29 June 2006 we attempted to extract rootworm larvae from roots of both non-infested ($n = 16$) and infested ($n = 16$) maize genotypes by drying samples within a greenhouse, which involved suspending root balls in mesh bags for 10 days and collecting escaping larvae from water-filled pans placed beneath samples. We recovered 0.06 ± 0.06 rootworm larvae per non-infested maize root ball, and 3.50 ± 0.57 rootworm larvae per infested maize ball. However, due to the time involved in processing samples, extracted larvae had begun to deteriorate, and therefore we were unable to assess associated bacteria. According to our soil growing degree day information, the majority of rootworm larvae would have left the maize roots and begun pupation in the surrounding soil at the time we collected root samples. For confirmation, on 20 July 2006 we sampled another set of roots from infested maize genotypes and attempted to extract rootworm larvae. However, no larvae were recovered from these field samples. Thus, we used 2nd and 3rd instar western corn rootworm larvae with no visible disease symptoms from a non-diapausing colony maintained at NCARL to explore the *Serratia* flora associated with rootworm larvae. Although rootworm eggs from a diapausing colony were used in field infestations, individuals from both colonies are reared using the same facilities and consume the same diet, therefore it is likely that they have a similar microbial community.

2.2. Microbiology

2.2.1. Culturing and enumeration of bacteria from soil, maize roots, and rootworm larvae

Soil and root samples from replicates 1 and 2 were processed on 20 July 2006, whereas samples from replicates 3 and 4 were kept in the refrigerator and processed on 21 July 2006. On 21 July 2006, four rootworm larvae samples were collected from the reared colony, surface-rinsed in deionized water and 70% ethanol, and processed. Each rootworm sample consisted of multiple rootworm larvae.

Sample processing was conducted using aseptic techniques and standard microbiological practices including use of a laminar flow hood. Protocols similar to those in the literature (e.g. Lambert et al., 1987; Baudoin et al., 2001) were used to culture and enumerate viable bacteria. The following samples were processed: a 5 g subsample from each maize root system (non-infested maize plants, $n = 16$; infested maize plants, $n = 16$), a 5 g subsample from each soil sample (non-infested soil, $n = 4$; infested soil, $n = 4$), and 4 rootworm larvae samples

(each sample = 1 g). Therefore, 32 maize root systems, 8 soil samples, and 4 rootworm larvae samples were processed. Samples were ground with a mortar and pestle, suspended in phosphate-buffered saline (1.18 g Na_2HPO_4, 0.22 g $NaH_2PO_4{\cdot}H_2O$, and 8.50 g NaCl per L; pH 7.5) with the non-ionic detergent Tween®80 (0.05% final concentration; Fisher Scientific), homogenized on a rotary shaker (10 min, 200 rpm), and dilution-plated (10^{-2} to 10^{-5}) onto caprylate-thallous agar (CT agar) in triplicate per standard microbiological procedures (Koch, 1994). Caprylate-thallous agar is made using thallous sulfate, and with the exception of *S. fonticola*, selectively isolates *Serratia* spp. (Starr et al., 1976; Grimont and Grimont, 2006). Colony forming units (CFU) were enumerated for each morphologically-distinct colony type (size, color, form, margin, elevation, texture, surface appearance) after 72 h incubation at 30 °C. CFU were expressed per gram dry weight of sample based on moisture contents of additional subsamples.

2.2.2. *Culturing of microorganisms from adult rootworms*

Similar protocols were used to isolate bacteria from field-collected adult rootworms with no apparent disease symptoms, although bacteria were not enumerated. Northern (*Diabrotica barberi* Smith and Lawrence; $n = 2♀, 2♂$) and southern corn rootworms (*D. undecimpunctata howardi* Barber; $n = 2♀, 2♂$) were collected from goldenrod and asters on 25 September 2006 near Beresford, SD. Insects were maintained in cages and fed artificial diet (F9768B-M, Bio-Serv, Frenchtown, NJ) and acorn squash (*Cucurbita pepo* L.) until processed on 11 October 2006.

Diseased rootworm adults were processed differently because we wanted to identify, not quantify, all pathogenic organisms. Bacteria from visibly diseased rootworms (interior and exterior of body was pinkish-red) from the NCARL rearing colony were isolated on three different dates. On 28 March 2006, one diseased western corn rootworm was macerated and plated onto an egg-agar media, then transferred to trypticase soy agar (TSA) and incubated at 37 °C. On 1 and 10 August 2007, bacteria from additional diseased rootworms (*D. barberi*, $n = 2$; *D. virgifera*, $n = 3$) were isolated. Each beetle was suspended in 1.0 mL sterile PBS, mashed using a sterile pestle, resuspended in an additional 1.0 mL PBS, and 0.1 mL plated onto the following solid media: TSA, R2A, potato dextrose agar (PDA), CT agar, and nutrient agar. Plates were incubated at 37 °C for 48–72 h and all visually distinct colonies isolated by restreaking on TSA media. Two visually distinct bacterial colonies from the original diseased rootworm and three visually distinct colonies from the five subsequent rootworms were isolated.

2.3. *Bacterial isolate identification*

Thirty bacterial colonies that had visually distinct cellular and colony morphology were recovered on solid media from all sample types (soil, maize roots, rootworm larvae, non-diseased and diseased rootworm adults). For 21 bacterial colonies that exhibited consistent colony morphology, one pure culture (isolate) was obtained by repeatedly streaking one representative colony onto CT or nutrient agar. For each of the remaining nine bacterial colonies that exhibited more variable colony morphology, two pure cultures (isolates) were obtained by repeatedly streaking a representative colony that originated from a different source. Therefore, a total of 39 bacterial isolates were cultured (Table 1). 16S rRNA gene sequences and phenotypic characterization were then used to determine the species identity of each isolate and expose redundancies caused by variations in cellular and colony morphology (i.e. size, color).

2.3.1. *16S rRNA gene sequence analysis*

DNA was extracted (Wizard® Genomic Purification kit, A1120; Promega, Madison, WI) and the nearly full-length 16S rRNA gene was amplified (50 μL reactions containing the following, all from Promega unless noted: 0.025 U/μL GoTaq DNA polymerase, 1× GoTaQ PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of forward and reverse primers, and 2% bovine serum albumin (Roche, Indianapolis, IN); PCR conditions: 2 min initial denaturation at 95 °C; 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 55 °C, and 1.5 min elongation at 72 °C; 5 min final elongation at 72 °C) using the primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). PCR products were purified (Wizard® PCR Purification kit, A2180) and then sequenced at the Iowa State Sequencing Facility with the primers 8F, 530F (5′-GTGCCAGCMGCCGCGG-3′), and 1100F (5′-GCAACGAGCGCAACCC-3′) (Lane, 1991). No chimeric sequences were identified using Chimera_Check ver. 2.7, RDP8.1, Bellerophon (Huber et al., 2004) and Mallard (Ashelford et al., 2006). Nearly full-length sequences were assembled within Bioedit and compared to the GenBank database using BLAST (Altschul et al., 1990). Sequence alignments, distance calculations, and cluster analysis (neighbor-joining method) were performed with ClustalW 1.83.

2.3.2. *Phenotypic characterization*

Although the 16S rDNA sequence is highly conserved in the Enterobacteriaceae and the genus *Serratia*, with 97% sequence similarity between *S. marcescens*, *S. rubidaea*, and *S. odorifera*, and over 99% sequence similarity among some members of the *S. liquefaciens–S. proteamaculans–Serratia grimesii* complex, *Serratia* species can be separated using 16S rDNA (Dauga et al., 1990; Spröer et al., 1999). However, to confirm biotype identity of *Serratia* isolates we used API®20E strips (bioMérieux, 2005) and phenotypic characterization tests (Grimont et al., 1981, 1982; Gerhardt et al., 1994; Grimont and Grimont, 2005, 2006), including: growth at 5 °C, growth at 40 °C, pigment production, carbohydrate utilization (*trans*-aconitate, benzoate, *meso*-erythritol, 4-hydroxybenzoate, *meso*-tartrate), acid produced from raffinose and xylose, arginine decarboxylase, esculine hydrolysis, and tetrathionate reduction.

2.4. *Statistics*

When bacterial colonies on agar plates were enumerated, certain colonies were easily separated into biotypes based on visually distinct cellular and colony morphology, especially pigmented and non-pigmented *Serratia* species. However, because it was difficult to definitively separate colonies of *Enterobacter*, *Pantoea*, and *Klebsiella*, colonies from these genera were combined into one biotype group for quantitative analyses, which was called Ent/Pant. Therefore, enumerations for the following biotype groups were used in statistical analyses: *S. marcescens* biotype A1b orange strain, *S. marcescens* biotype A1b pink strain, *S. marcescens* biotype A4, all biotypes

Table 1 – Identification of isolates using 16S rRNA gene sequences

Isolate ID	GenBank accession#	Genus/species/biotype	Closest cultured match, GenBank accession	% Similar (ca. 1460 bases)
DAP1[a]	EU302826	*Serratia grimesii*	*S. grimesii* AJ233430	98.0
DAP2[a]	EU302827	*S. grimesii*	*S. grimesii* AJ233430	97.2
DAP3[a]	EU302828	*S. grimesii*	*S. grimesii* AJ233430	88.7[b]
DAP4[a]	EU302829	*S. grimesii*	*S. grimesii* AJ233430	99.2
DAP5[a]	EU302830	*S. grimesii*	*S. grimesii* AJ233430	98.8
DAP6[a]	EU302831	*S. grimesii* ACD	*S. proteomaculans* J233435	99.5
DAP7[a]	EU302832	*S. grimesii*	*S. proteomaculans* J233435	98.9
DAP8	EU302833	*Salmonella typhimurium*	*S. typhimurium* AF170176	96.7
DAP9	EU302834	*Enterobacter* sp.	*E. aerogenes* AF395913	91.2
DAP10	EU302835	*Enterobacter* sp.	*E. aerogenes* AF395913	91.4
DAP11	EU302836	*Enterobacter* sp.	*E. aerogenes* AF395913	96.0
DAP12	EU302837	*Pantoea agglomerans*	*P. agglomerans* AY335552	99.7
DAP13	EU302838	*P. agglomerans*	*P. agglomerans* AY335552	99.1
DAP14	EU302839	*P. agglomerans*	*P. agglomerans* AY335552	98.7
DAP15	EU302840	*P. agglomerans*	*P. agglomerans* AY335552	99.5
DAP16	EU302841	*P. agglomerans*	*P. agglomerans* AY335552	99.5
DAP17	EU302842	*P. agglomerans*	*P. agglomerans* AY335552	99.7
DAP18	EU302843	*P. agglomerans*	*P. agglomerans* AY335552	99.3
DAP19	EU302844	*E. aerogenes*	*E. aerogenes* AF395913	98.9
DAP20	EU302845	*P. agglomerans*	*P. agglomerans* AY941834	99.0
DAP21	EU302846	*E. aerogenes*	*E. aerogenes* AJ251468	99.2
DAP22	EU302847	*Kluyvera ascorbata*	*K. ascorbata* AJ627203	98.6
DAP23	EU302848	*Klebsiella pneumoniae*	*K. pneumoniae* X87276	99.3
DAP24	EU302848	*Kle. pneumoniae*	*K. pneumoniae* CP000647	99.0
DAP25[a]	EU302850	*Serratia plymuthica*	*S. entomophila* AJ233427	99.0
DAP26[a]	EU302851	*Serratia marcescens*	*S. marcescens* AJ550467	99.4
DAP27[a]	EU302852	*S. marcescens* A1b P	*S. marcescens* AB061685	99.6
DAP28[a]	EU302853	*S. marcescens* A1b P	*S. marcescens* AB061685	99.5
DAP29[a]	EU302854	*S. marcescens* A1b P	*S. marcescens* AB061685	99.7
DAP30[a]	EU302855	*S. marcescens* A1b P	*S. marcescens* AB061685	99.9
DAP31[a]	EU302856	*S. marcescens* A4	*S. marcescens* AB061685	99.6
DAP32[a]	EU302857	*S. marcescens* A1b O	*S. marcescens* AB061685	99.5
DAP33[a]	EU302858	*S. marcescens* A1b O	*S. marcescens* AB061685	99.5
DAP34[a]	EU302859	*S. marcescens* A1b O	*S. marcescens* AB061685	99.4
DAP35[a]	EU302860	*S. marcescens* A1b O	*S. marcescens* AB061685	99.5
DAP36[c]	EU302861	*S. marcescens*	*S. marcescens* AB061685	99.4
DAP37[d]	EU302862	*Pseudomonas aeruginosa*	*P. noraviensis* AY970952	98.4
DAP38	EU302863	*Ps. aeruginosa*	*P. aeruginosa* EF064786	99.9
DAP39	EU302864	*Lactococcus lactis*	*L. lactis* AY626141	98.8

[a] Identifications are combined result of sequence analysis and phenotypic testing (Tables 2 and 3).
[b] Sequence quality poor.
[c] Isolate had orange pigmentation similar to the *S. marcescens* A1b O strain.
[d] Species designation based on API®20E strips.

of *S. grimesii*, and all *Enterobacter/Pantoea/Klebsiella* species. Only colonies that could be unambiguously assigned to these groups were used in statistical analyses. Uncommon biotype groupings were not used in quantitative analyses. For soil, maize roots, and rootworm larvae samples, colony-forming units (CFUs) of each biotype group were reported per g dry weight of sample.

For comparisons between different sample types (i.e. soil, roots), biotype enumeration data were converted to percentages of total CFUs. These data were analyzed using GLM (SYSTAT®; SPSS Inc., 1998) with infestation status as the whole plot treatment, sample origin as the subplot treatment, and percentage of total CFUs as the dependent variable. The whole plot error was used in testing effects of infestation status, while the subplot error was used in testing effects of sample origin and the interaction between the two independent variables. To investigate impacts of maize genotype and rootworm infestation status on specific bacterial groups, CFU data from maize roots were log(X + 1) transformed and analyzed using GLM (SYSTAT®; SPSS Inc., 1998) with infestation status as the whole plot treatment, maize genotype as the subplot treatment, and bacterial CFU density as the dependent variable. The whole plot error was used in testing effects of infestation status, while the subplot error was used in testing effects of maize genotype and the interaction between the two independent variables. To investigate differences in densities of all bacterial groups within a specific environment, CFU data from non-infested maize roots, infested maize roots, and larval rootworms were log(X + 1) transformed and analyzed using ANOVA in SYSTAT® (SPSS Inc., 1998), with bacterial identity as the independent variable and CFU density as the dependent variable, followed by Fisher's Least-Significant Difference post hoc test.

Because of high variance in the data, we converted bacterial CFU data to binary (presence/absence) data and used multivariate cluster analysis in SYSTAT® (SPSS Inc., 1998) with hierarchical clustering, complete linkage method (farthest neighbor), and clustering distances calculated using a 1-Pearson correlation coefficient distance metric to explore how similarities in overall bacterial profiles were related to sample origin.

3. Results

3.1. *Isolate identification*

Based on their 16S rRNA sequences, 19 of the 39 isolates were identified as *Serratia* species (Table 1; Fig. 1). The 16S rRNA gene sequences of seven isolates (DAP1-7) were highly similar to GenBank data entries for *S. liquefaciens*, *S. proteamaculans*, and *S. grimesii*, although, due to the extremely high similarity between the 16S rRNA sequences of this group (Spröer et al., 1999), phenotypic characterization tests were used to confirm species and biotype identity (Table 2). Eleven isolates (DAP26-36) were identified as *S. marcescens* (Tables 1 and 3). The non-pigmented isolate DAP31 was identified as *S. marcescens* biotype A4 (Table 3; Fig. 1). Pigmented isolates of *S. marcescens* biotype A1 (DAP27-35) were identified as A1b rather than A1a because they did not utilize *meso*-tartrate (Grimont and Grimont, 2005, 2006). Isolates from the *S. marcescens* biotype A1b group were separated into two empirically defined strains based on pigmentation variations. Four of these isolates (DAP32-35) had distinctive orange pigmentation (O) during early colony growth, while the other four (DAP27-30) lacked this pigmentation and appeared bright reddish-pink (P).

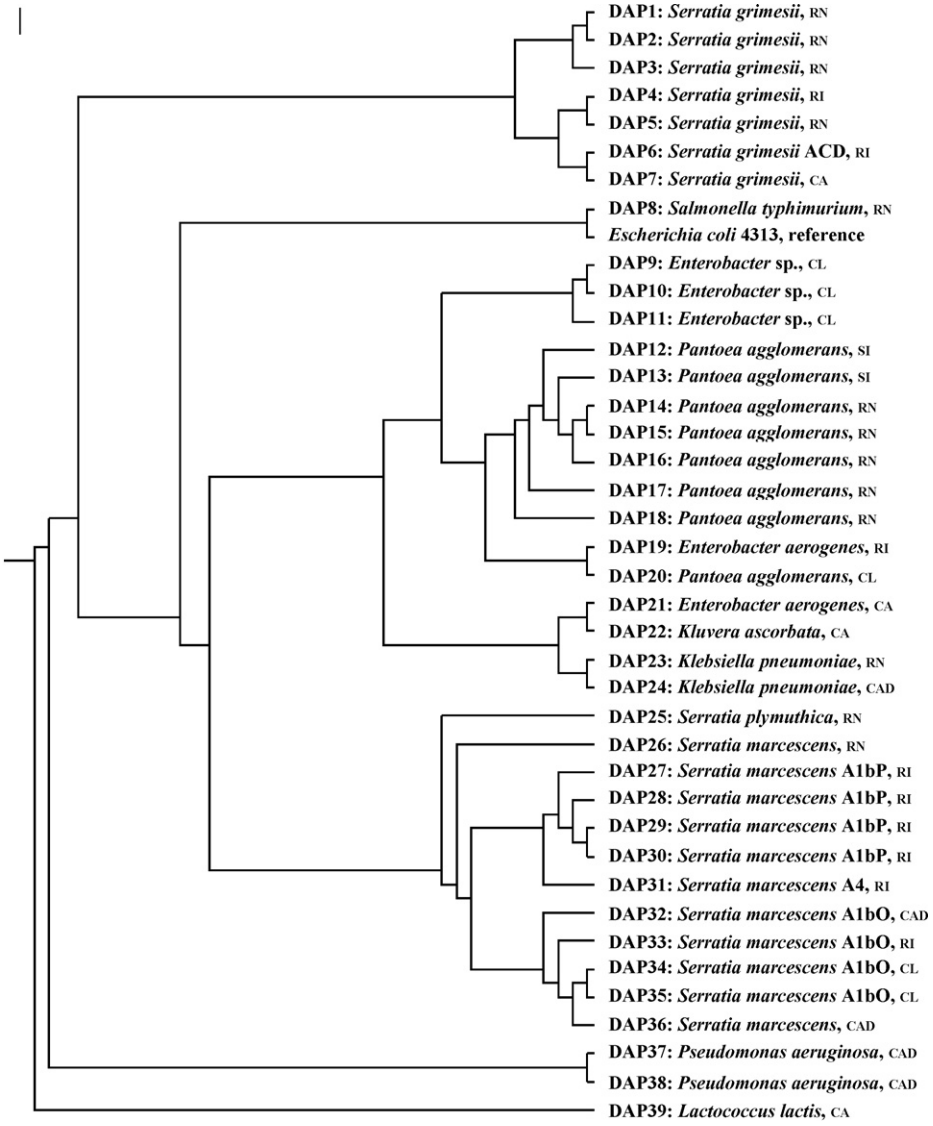

Fig. 1 – Dendogram (neighbor-joining, no branch length) of the 16S rRNA gene sequences of bacterial isolates showing isolate identification (DAP numbers, also see Table 1) and final genus/species/biotype/strain determination. *Escherichia coli* strain 4313 was used as a reference in sequence analyses. Capital letters following the species name indicate isolate origin: CA: non-diseased adult beetle, CAD: diseased rootworm, CL: rootworm larvae, RN: non-infested maize root, RI: infested maize root, SI: infested bulk soil.

Table 2 – Phenotypic identification of *Serratia grimesii* and *S. plymuthica* biotypes

ID#[a]	DAP1	DAP2	DAP3	DAP4	DAP5	DAP6	DAP7	DAP25
Final ID/biotype[b]	Sgri	Sgri	Sgri	Sgri	Sgri	Sgri ACD	Sgri	Sply
Phenotypic characterization tests[c]								
5 °C	+	+	+	+	+	+	+	+
40 °C	–	–	–	–	–	–	–	–
RAF	+		+		+	+	+	+w
XYL	+		+		+	+	+	+
PIGM	–	–	–	–	–	–	–	–
ACO	–	–	–	–	–	+	–	±
BEN	–	–	–	–	–	–	–	–
ERY	–	–	–	–	–	–	–	–
HB	+	+	–	–	±	±	±	+
TAR	–	–	–	–	–	–	–	–
TET	+	+	+	+	+	+	+	–
ESC	+	+	+	+	+	+	+	+
ARG	+	+	+	+	+	+	+	–
API® 20E tests[d]								
ONPG	+		+		+	+	+	+
ADH	–		–		+	±	–	–
LDC	+		+		+	+	+	–
ODC	+		+		+	+	+	–
CIT	+		+		+	+	+	+
H2S	–		–		–	–	–	–
URE	–		–		–	–	–	–
TDA	–		–		–	–	–	–
IND	–		–		–	–	–	–
VP	–		–		+	–	–	–
GEL	+		+		+	+	+	–
GLU	+		+		+	+	+	+
MAN	+		+		+	+	+	+
INO	+		+		±	±	+	+
SOR	+		+		+	+	+	–
RHA	–		–		–	–	–	–
SAC	+		+		+	+	+	+
MEL	+		+		–	±	+	–
AMY	+		+		+	+	+	+
ARA	+		+		±	+	+	–
OX	–	–	–	–	–	–	–	–

[a] From Fig. 1.
[b] Sgri: *S. grimesii*, Sply: *S. plymuthica*. Unlabeled Sgri are either atypical ACD or atypical C1d biotypes.
[c] Growth at 5 °C, growth at 40 °C, RAF: acid from raffinose, XYL: acid from xylose, PIGM: production of colored pigment, ACO: *trans*-aconitate use, BEN: benzoate use, ERY: *meso*-erythritol use, HB: 4-hydroxybenzoate use, TAR: *meso*-tartrate use, TET: tetrathionate reduction, ESC: esculine hydrolysis, ARG: arginine decarboxylase; w: weak reaction.
[d] ONPG: β-galactosidase, ADH: arginine dehydrolase, LDC: lysine decarboxylase, ODC: ornithine decarboxylase, CIT: citrate utilization, H2S: H_2S production, URE: urease, TDA: tryptophane deaminase, IND: indole production, VP: Voges Proskauer, GEL: gelatinase, GLU: D-glucose use, MAN: mannitol use, INO: inositol use, SOR: D-sorbitol use, RHA: L-rhamnose use, SAC: D-sucrose use, MEL: D-melibiose use, AMY: amygdalin use, ARA: L-arabinose use, OX: cytochrome-oxidase.

Orange and pink variants of prodigiosin-producing *S. marcescens* have previously been recorded (Rizki, 1954; Williams and Green, 1956), and changes in pigmentation are related to several environmental conditions such as temperature, culture media (Williams et al., 1971), and pH (Allen, 1967; Solé et al., 1994). The 16S rRNA sequences supported the division of the *S. marcescens* biotype A1b group, with the orange strains clearly separated from the pink strains. For two isolates (DAP25-26), 16S rRNA gene sequences and phenotypic characterization did not concur (Tables 1–3). DAP26 was tenuously identified as *S. marcescens*, and DAP25 was tentatively identified as *Serratia plymuthica*.

Although CT agar is selective for *Serratia*, 17 morphologically distinct isolates (DAP8-23, 39) representing other genera were recovered using this media and identified using their 16S rRNA gene sequences, including *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Kluyvera ascorbata*, *Lactococcus lactis*, *Pantoea agglomerans*, and *Salmonella typhimurium* (Table 1; Fig. 1). Some *E. aerogenes* and *E. cloacae* strains have previously been isolated on CT agar (Starr et al., 1976), and certain *Acinetobacter*, *Providencia*, and *Pseudomonas* spp. occasionally grow on this media (Grimont and Grimont, 2006). The similarity of the 16S rRNA gene sequences of these non-*Serratia* isolates among themselves, the 17 *Serratia* isolates originally recovered from CT agar, and five isolates associated with diseased rootworm adults originally recovered from multiple types of solid media (DAP24, 32, 36–38) are shown in Fig. 1. All 16S rRNA gene sequences were

Table 3 – Phenotypic identification of *Serratia marcescens* biotypes

ID#[a]	DAP 26	DAP 27	DAP 28	DAP 29	DAP 30	DAP 31	DAP 32	DAP 33	DAP 34	DAP 35
Final ID/ biotype[b]	Smar	Smar A1bP	Smar A1bP	Smar A1bP	Smar A1bP	Smar A4	Smar A1bO	Smar A1bO	Smar A1bO	Smar A1bO
Phenotypic characterization tests[c]										
5 °C	–	–	–	–	–	–	–	–	–	–
40 °C	–	+	+	+	+	+	+	+	+	+
RAF	+	+	+	+	+	+w	+	+	+	+
XYL	+	–	–	–	–	–w	+	–	–	–
PIGM	–	+	+	+	+	–	+	+	+	+
ACO										
BEN	–	+	+	+	+	–	+	+	+	+
ERY	–	+	+	+	+	+	+	+	+	+
HB	–	–	–	–	–	+	–	–	–	–
TAR	–	–	–	–	–	–	–	–	–	–
TET	–	+	+	+	+	–	+	+	+	+
ESC										
ARG										
API® 20E tests[d]										
ONPG	+	+	+	+	+	+	+	+	+	+
ADH	–	–	–	–	–	–	–	–	–	–
LDC	–	+	+	+	+	+	+	+	+	+
ODC	–	+	+	+	+	+	+	+	+	+
CIT	+	+	+	+	+	+	+	+	+	+
H2S	–	–	–	–	–	–	–	–	–	–
URE	–	–	–	–	–	+	–	–	–	–
TDA	–	–	–	–	–	–	–	–	–	–
IND	–	–	–	–	–	–	–	–	–	–
VP	–	+	+	+	+	–	–	+	+	+
GEL	–	+	+	+	+	+	+	+	+	+
GLU	±	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	±	+	+	+	+	±
INO	–	±	±	±	±	+	+	+	+	+
SOR	–	+	+	+	+	+	+	+	+	+
RHA	–	–	–	–	–	–	–	–	–	–
SAC	–	+	±	±	±	+	+	+	+	+
MEL	–	+	+	+	+	+	+	+	+	+
AMY	–	±	±	±	+	+	+	±	±	+
ARA	–	±	+	+	+	+	+	±	±	+
OX	–	–	–	–	–	–	–	–	–	–

[a] From Fig. 1.
[b] Smar: *S. marcescens*, O: orange strain, P: pink strain.
[c] Growth at 5 °C, growth at 40 °C, RAF: acid from raffinose, XYL: acid from xylose, PIGM: production of colored pigment, ACO: *trans*-aconitate use, BEN: benzoate use, ERY: *meso*-erythritol use, HB: 4-hydroxybenzoate use, TAR: *meso*-tartrate use, TET: tetrathionate reduction, ESC: esculine hydrolysis, ARG: arginine decarboxylase; w: weak reaction.
[d] ONPG: β-galactosidase, ADH: arginine dehydrolase, LDC: lysine decarboxylase, ODC: ornithine decarboxylase, CIT: citrate utilization, H2S: H_2S production, URE: urease, TDA: tryptophane deaminase, IND: indole production, VP: Voges Proskauer, GEL: gelatinase, GLU: D-glucose use, MAN: mannitol use, INO: inositol use, SOR: D-sorbitol use, RHA: L-rhamnose use, SAC: D-sucrose use, MEL: D-melibiose use, AMY: amygdalin use, ARA: L-arabinose use, OX: cytochrome-oxidase.

deposited in GenBank under accession numbers EU302826–EU302864.

3.2. *Differences in bacteria associated with bulk soil and maize roots*

Bacteria recovered on CT agar from bulk soil were primarily *Enterobacter/Pantoea/Klebsiella* spp. that were also commonly associated with maize roots, resulting in non-significant *P*-values for all effects (Table 4; Fig. 2). Both orange and pink strains of *S. marcescens* A1b were absent from bulk soil, and significant interactions (A1bO, $P = 0.06$; A1bP $P < 0.001$) between sample origin (from bulk soil or maize roots) and infestation with rootworms indicated that these biotypes were associated with maize roots, but primarily when soil was infested with rootworm eggs (Table 4; Fig. 2). No *Serratia* species were isolated from non-infested bulk soil samples, and *S. grimesii* was only occasionally associated with bulk soil that was infested with rootworm eggs. Infestation status did not impact the percentage of *S. marcescens* A4 and *S. grimesii* CFUs associated with maize roots (Table 4; Fig. 2). While other bacterial colonies grew on CT agar inoculated with soil samples, these isolates did not grow after continuous replating on this media, and may have initially been utilizing the small amount of Tween®80 (0.05%), or more likely, organic material present in the original soil inoculum.

Table 4 – Statistical information for effects of sample origin and infestation status on percentages of total bacterial CFUs associated with bulk soil and maize roots

Effect	d.f.	F-value	P-value
Serratia marcescens A1b orange			
Origin	1.6	9.30	0.023
Infestation	1.6	5.30	0.061
Origin × infestation	1.6	5.30	0.061
Serratia marcescens A1b pink			
Origin	1.6	71.75	<0.001
Infestation	1.6	57.21	<0.001
Origin × infestation	1.6	57.21	<0.001
Serratia marcescens A4			
Origin	1.6	8.52	0.027
Infestation	1.6	1.65	0.247
Origin × infestation	1.6	1.65	0.247
Serratia grimesii			
Origin	1.6	21.91	0.003
Infestation	1.6	1.47	0.271
Origin × infestation	1.6	0.20	0.668
Enterobacter spp.			
Origin	1.6	3.68	0.104
Infestation	1.6	0.01	0.938
Origin × infestation	1.6	0.01	0.930

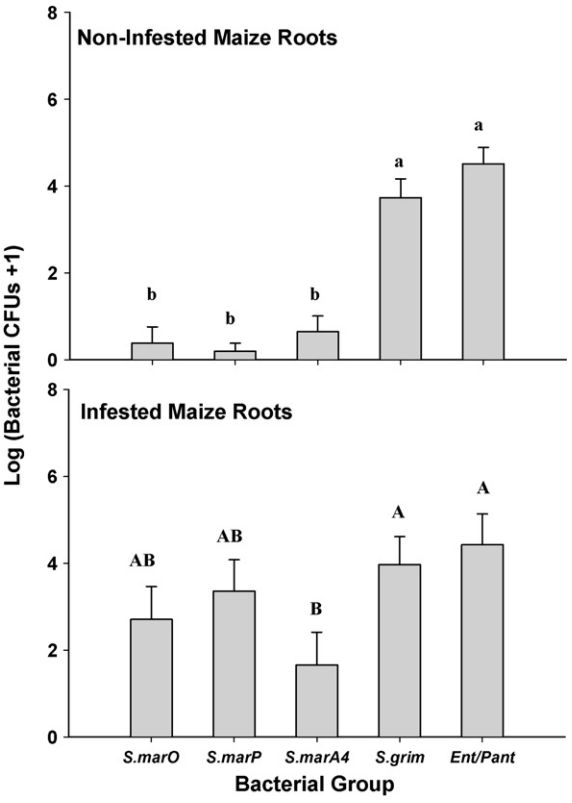

Fig. 3 – Bacterial group densities associated with non-infested and infested maize roots. *SmarO*: *Serratia marcescens* biotype A1b orange strain, *S.marP*: *S. marcescens* biotype A1b pink strain, *S.marA4*: *S. marcescens* biotype A4, *S. grimesii*: all biotypes, *Ent/Pant*: *Enterobacter*, *Pantoea*, and *Klebsiella* spp. Data are means ± standard error of the mean, and averaged across all maize genotypes. Lower case letters indicate comparisons between non-infested maize roots, while upper case letters indicate comparisons between infested maize roots.

3.3. *Maize roots: effects of genotype and infestation on bacterial density*

S. typhimurium (DAP8) was commonly associated with maize roots regardless of infestation status or genotype, but the densities of this species were not quantified. The unidentified *S. marcescens* biotype (DAP26) and *S. plymuthica* were rare, with the former only associated with non-infested roots of maize genotypes NGSDCRW1(S2)C4 and BS29-11-01 in one replicate,

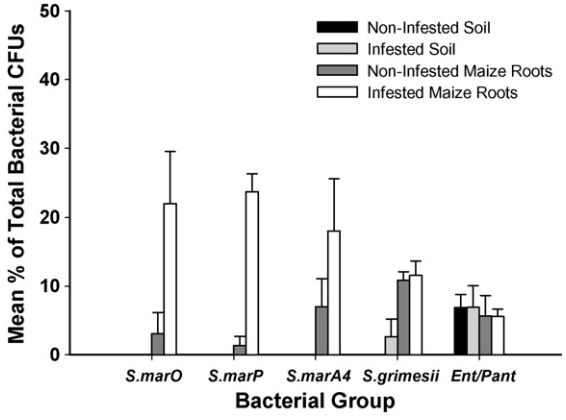

Fig. 2 – Mean percent (±standard error of the mean) of total colony forming units (CFUs) for bacterial groups associated with non-infested and infested soil and maize roots. *SmarO*: *Serratia marcescens* biotype A1b orange strain, *S.marP*: *S. marcescens* biotype A1b pink strain, *S.marA4*: *S. marcescens* biotype A4, *S. grimesii*: all biotypes, *Ent/Pant*: *Enterobacter*, *Pantoea*, and *Klebsiella* spp. Data averaged across all maize genotypes.

and the latter only isolated from non-infested roots of CRW3(C6).

Overall, maize genotype did not impact density of any bacterial group (All groups: *Maize*, d.f. = 3.18, $P > 0.10$; *Maize* × *Infestation*, d.f. = 3.18; $P > 0.10$). *S. grimesii* and *Enterobacter/Pantoea/Klebsiella* spp. were frequently associated with maize roots, and infestation with rootworm eggs did not have a significant impact on their densities (Both groups: *Infestation*, d.f. = 1.6, $P > 0.10$; Fig. 3). Additionally, these two groups were more abundant than all other bacterial groups on non-infested roots (All comparisons: $P < 0.0001$) and more abundant than *S. marcescens* A4 on infested maize roots (*S. grimesii*: $P = 0.045$; *Ent/Pant*: $P = 0.019$; Fig. 3). Although infestation with rootworm eggs did not have a significant impact on densities of non-pigmented *S. marcescens* A4 (*Infestation*, d.f. = 1.6, $P = 0.25$), densities of both pigmented *S. marcescens* A1b strains were higher on infested roots compared to non-infested roots, although the *P*-value for the orange strain was marginally significant (Orange A1b strain: *Infestation*, d.f. = 1.6, $P = 0.061$;

Pink A1b strain: *Infestation*, d.f. = 1.6, $P = 0.0003$). There were no differences between the numbers of *S. marcescens* A1b orange and *S. marcescens* A1b pink in either non-infested ($P = 0.69$) or infested maize roots ($P = 0.55$; Fig. 3).

3.4. Bacteria associated with rootworms

There were significant differences among the densities of bacteria groups enumerated from rootworm larvae with no obvious disease symptoms (d.f. = 4.15; $P < 0.0001$), with *Enterobacter/Pantoea/Klebsiella* spp. being the most abundant bacterial group (Fig. 4). The *S. marcescens* A1b orange strain was the most abundant type of *Serratia*, and had higher numbers than *S. marcescens* A1b pink strain ($P = 0.029$). *S. marcescens* A4 and *S. grimesii* were not isolated from rootworm larvae.

Three bacteria were isolated from non-diseased adult rootworms (Tables 1 and 5). *S. grimesii* (DAP7) was isolated from a male southern corn rootworm, *L. lactis* (DAP39) was isolated from a female northern corn rootworm, and *K. ascorbata* (DAP 22) was isolated from a male northern corn rootworm. However, bacteria within these specimens may have been impacted by the presence of an antibiotic in their diet prior to sample processing or ingestion of ethanol during surface sterilization.

Despite using an array of growth media, including non-selective types (NA, TSA), only three different bacterial species were recovered from diseased adult rootworms. *Pseudomonas aeruginosa* (DAP37) and the orange *S. marcescens* A1b strain (DAP32) were isolated from the visibly diseased rootworm adult processed on 28 March 2006, while *K. pneumoniae* (DAP24), *P. aeruginosa* (DAP38), and *S. marcescens* (DAP36) were isolated from diseased rootworms processed on 1 and 10 August 2007 (Tables 1 and 5). While the latter *S. marcescens* isolate (DAP36) was not subjected to phenotypic testing, it produced an orange pigment during early colony growth, and was likely the orange strain of *S. marcescens* biotype A1b, an affiliation supported by the similarity in 16S rRNA gene sequence alignments (Fig. 1). Fungal colonies present on nutrient agar and potato-dextrose agar were identified as *Alternaria* and *Aspergillus* species (data not shown).

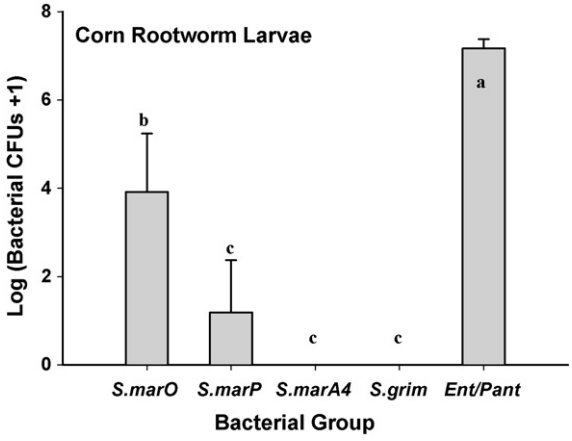

Fig. 4 – Bacterial group densities associated with non-diseased western corn rootworm larvae. *S.marO*: *Serratia marcescens* biotype A1b orange strain, *S.marP*: *S. marcescens* biotype A1b pink strain, *S.marA4*: *S. marcescens* biotype A4, *S. grimesii*: all biotypes, *Ent/Pant*: *Enterobacter*, *Pantoea*, and *Klebsiella* spp. Data are means ± standard error of the mean.

3.5. Bacterial community cluster analysis

Cluster analysis was used to investigate similarities in bacterial profiles of samples from different origins (data not

Table 5 – Summary of bacteria isolated from *Diabrotica* spp. lacking disease symptoms and from visibly diseased adults

Bacteria	Isolate ID[a]	Diseased[b]	Life stage[c]	Species[d]
Enterobacter sp.	DAP9, DAP10, DAP11	N	L	WCR
Enterobacter cloacae	[e]	N	L	WCR
Klebsiella pneumoniae	DAP24	Y	A	NCR and WCR
Kluyvera ascorbata	DAP22	N	A	NCR
Lactococcus lactis	DAP39	N	A	NCR
Pantoea agglomerans	DAP20	N	L	WCR
Pseudomonas aeruginosa	DAP37, DAP38	Y	A	WCR
Serratia grimesii	DAP7	N	A	SCR
Serratia marcescens[f]	DAP36	Y	A	WCR
Serratia marcescens A1b orange	DAP32[g]	Y	A	NCR and WCR
Serratia marcescens A1b orange	DAP34, DAP35	N	L	WCR
Serratia marcescens A1b pink	[g]	N	L	WCR

[a] DAP9 and DAP10 were isolated from the same rootworm larvae sample, DAP11 and DAP 20 were isolated from the same rootworm larvae sample, DAP34 and DAP35 were isolated from the same rootworm larvae sample, DAP32 and DAP37 were isolated from the same diseased WCR adult.
[b] Y: diseased with visible red or pink color, N: apparently non-diseased with normal coloration.
[c] A: adult, L: larvae.
[d] NCR: northern corn rootworm, *Diabrotica barberi* Smith and Lawrence; SCR: southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber; WCR: western corn rootworm, *Diabrotica virgifera virgifera* LeConte.
[e] Identified solely using API® 20E strips.
[f] No phenotypic tests performed on this isolate, which is likely *S. marcescens* A1b orange based on visual observations.
[g] Bacterial strain identified by visual comparison with known strain from a different origin.

shown). In general, the bacterial community associated with both non-infested and infested soil clustered together, and appeared to be most closely related to bacterial profiles of non-infested maize roots. Infestation status appeared to influence bacterial profiles of maize roots to a greater degree than maize genotype. While there was still variability of bacterial communities within all root samples, non-infested roots tended to cluster together with soil, while infested maize roots tended to cluster together with rootworm larvae. Bacterial profiles of non-infested roots tended to be more variable than infested roots, with five samples grouped with infested roots and rootworms, while only two infested root samples were similar to non-infested roots.

4. Discussion

We found that (i) *Serratia* species diversity and density were enriched in the maize rhizosphere compared to the surrounding bulk soil; (ii) there was no effect of corn genotype on *Serratia* and related bacteria in the maize rhizosphere; and (iii) infestation with western corn rootworm eggs had a significant positive effect on the density of specific *Serratia* species in the maize rhizosphere, including one biotype (*S. marcescens* A1bO) that we repeatedly isolated from diseased adult rootworms.

Although *Serratia* are generally associated with soil (Grimont and Grimont, 2006), we rarely isolated culturable *Serratia* species from bulk soil. In contrast, *Enterobacter/Pantoea/Klebsiella* species were associated with bulk soil and maize roots. In general, bulk soil has lower species diversity and total numbers of microorganisms compared to the plant rhizosphere (Baudoin et al., 2001; O'Flaherty et al., 2003; Brusetti et al., 2004). Less information is available regarding the microbial diversity present within a genus (i.e. *Serratia*) or family (i.e. Enterobacteriaceae). Although less than 1% of soil bacteria can routinely be cultured (Schloss and Handelsman, 2004, 2006), meta-analyses of 16S rRNA gene clone libraries show that the class Gammaproteobacteria may comprise roughly 10% of soil bacteria (Janssen, 2006). Consequently, species within the family Enterobacteriaceae likely comprise about 5% bacteria within the soil.

We identified three *Serratia* species associated with maize roots: *S. marcescens*, *S. grimesii*, and *S. plymuthica*. *S. marcescens* has been isolated from the rhizosphere of several plants, including citrus trees (Queiroz and Melo, 2006) and rice (Gyaneshwar et al., 2001), although we could not find a definitive report of *S. marcescens* A1b in the maize rhizosphere. While some *S. marcescens* are plant pathogens (Bruton et al., 2003; Rascoe et al., 2003), other *S. marcescens* strains are beneficial to plants and can promote plant growth by suppressing plant-pathogenic fungi (Ordentlich et al., 1988; Someya et al., 2005), or by induction of systemic plant resistance (Press et al., 1997, 2001). Non-pigmented *S. marcescens* A4 and the pigmented biotype A1b were occasionally associated with non-infested maize roots. The A4 biotype is often associated with infections in hospital patients (Grimont and Grimont, 1978; Gargallo-Viola, 1989), but is also associated with plants (Grimont et al., 1981), other natural environments (Grimont and Grimont, 2006), and various insects (Grimont et al., 1979; El-Sanousi et al., 1987). *S. marcescens* A1b has been isolated from figs and fig wasps (Grimont et al., 1981; Grimont and Grimont, 2006), water, and soil (Gargallo-Viola, 1989; Grimont and Grimont, 2006).

S. grimesii was commonly associated with maize roots. This species has been previously isolated from the rhizosphere of several plant species (Grimont et al., 1981), including strawberry, (oilseed rape (Berg et al., 2002), and non-transgenic potato (Lottmann et al., 1999). Although *S. liquefaciens*, a closely-related species, is associated with maize roots (Lambert et al., 1987; McInroy and Kloepper, 1995), with one strain linked to increases in maize dry weight and yield (Lalande et al., 1989), to our knowledge *S. grimesii* has not previously been isolated from this environment. *S. grimesii* strains have been reported to function as plant growth promotors by having antagonistic action against plant-pathogenic fungi. We also isolated *S. grimesii* (DAP7) from non-diseased adult southern corn rootworms, although the microbe could have been acquired during consumption of maize roots (Tran and Marrone, 1988). *S. grimesii* has previously been isolated from insects, including dead European spruce bark beetles (Yilmax et al., 2006), although strains of this species were not pathogenic to spiny and pink bollworm larvae (Khoja et al., 2006) or New Zealand grass grubs (Glare et al., 1993).

S. plymuthica was infrequently isolated in our study, and was associated with non-infested maize roots. However, we only collected maize roots on one date during the growing season (approximately 60 days after plant emergence), and so this species may have been more abundant earlier or later in the season. Plant maturity can affect the maize rhizobacteria flora (Lambert et al., 1987; Buyer and Kaufman, 1996; Brusetti et al., 2004), with community structure stabilizing after 38 d of plant growth (Chiarini et al., 1998). Buyer and Kaufman (1996) found that the density of a group of enteric maize rhizobacteria that included *Serratia* tended to increase from June to August, although the effect was not significant. *S. plymuthica* has previously been isolated from plants (Grimont et al., 1981), the rhizosphere of oilseed rape (Kalbe et al., 1996), potato (Lottmann et al., 1999), sugar beet roots (Ashelford et al., 2002), maize (McInroy and Kloepper, 1995; Stock et al., 2003), and banded cucumber beetle adults (Schalk et al., 1987), and is known to exhibit plant-growth promoting properties (Berg, 2000; Kurze et al., 2001; Berg et al., 2005a, 2006).

Maize genotype did not influence the abundance or diversity of bacteria (isolated on CT agar) from maize roots, which parallels studies on potato (Lottmann et al., 1999) and maize (Chiarini et al., 1998; Gomes et al., 2001; Schmalenberger and Tebbe, 2003; Fang et al., 2005). However, other researchers have found dissimilar rhizobacterial communities associated with maize cultivars planted in the same field (Lambert et al., 1987) and non-transgenic versus transgenic maize roots grown in the greenhouse (Brusetti et al., 2004). In addition, Miller et al. (1989) found differences in the total number of bacteria associated with the rhizosphere of maize cultivars with differential susceptibility to plant-pathogenic *Fusarium* fungi.

Infestation with rootworms eggs positively influenced the abundance of both orange and pink strains of *S. marcescens* A1b associated with maize roots. However, these two *S. marcescens* strains were also associated with rootworm larvae from a reared colony. Thus, it is unclear if rootworm larvae hatching from *Serratia*-infested eggs introduced the orange and pink strains of *S. marcescens* A1b into maize roots, or if changes in

the rhizosphere after infestation encouraged the growth of these *Serratia* strains. *Serratia* are often associated with multiple insect developmental stages, and can be transmitted maternally from females to offspring, and horizontally between members of the same generation (Iverson et al., 1984; Sikorowski and Lawrence, 1998; Sikorowski et al., 2001; Moran et al., 2005). Therefore, if rootworm eggs from the NCARL rearing colony were infected with *S. marcescens*, larvae that emerged in the field could have harbored these microbes. Pink and red eggs have been observed within the rearing colony, and we isolated the orange strain of *S. marcescens* A1b from rootworm larvae and diseased adults. Although the majority of rootworm larvae would have crawled away from the maize roots and begun pupation in the surrounding soil when roots were collected from the field, it is possible that some infected larvae, exuviae, and/or frass could have remained inside root samples, or that larvae could have contaminated the root tissue while feeding and/or tunneling inside roots. Rhizobacteria can be isolated from insect frass (Snyder et al., 1999), and rootworms can harbor and vector rhizobacteria (Snyder et al., 1998) and plant pathogens such as *Fusarium* (Palmer and Kommedahl, 1969; Gilbertson et al., 1986). Because the field was infested with rootworm eggs originating from the rearing colony, the *Serratia* we isolated from reared larvae likely parallels that of larvae that hatched in the field. However, while rootworm larvae from the colony had higher densities of the orange strain of *S. marcescens* A1b, which was the only strain isolated from diseased rootworm adults, comparable densities of orange and pink strains were isolated from infested roots. Thus, the association of the orange *S. marcescens* A1b strain with infested roots may be linked to the activity of infected rootworms hatching from contaminated eggs, but the origin of the pink *S. marcescens* A1b strain isolated from infested maize roots is less certain. Further research is needed to confirm the origin of *S. marcescens* strains within infested roots. It is possible that the presence of the pink strain could be due to plant-mediated changes in the rhizosphere after infestation, because low levels of both biotypes were associated with non-infested roots. However, this could be related to the presence of non-rootworm arthropods, contamination by drops of rootworm-infested agar, or movement of larval rootworms through the soil, although the latter two scenarios are unlikely due to the presence of non-infested buffer plants between treatment rows and at the end of infested blocks.

Because the *S. marcescens* A1b orange strain was consistently associated with diseased rootworm adults, it was likely entomopathogenic. *P. aeruginosa* (DAP37, 38) and *K. pneumoniae* (DAP 24) were also consistently isolated from diseased adult rootworms and are well-known opportunistic pathogens that likely interact with *S. marcescens* A1bO in causing disease in adult rootworms. Functions of other isolated *Serratia* spp., such as the *S. grimesii* isolated from non-diseased adult rootworms, are unknown. Some strains of *Serratia* associated with insects are symbionts that can detoxify ingested plant compounds (Dillon and Dillon, 2004), play a role in host nutrition (Iverson et al., 1984), and affect host fitness and development (Graber and Breznak, 2005; Moran et al., 2005). In addition, *Serratia* strains isolated from abdomens of female western corn rootworms may function as ovipositional cues because female rootworms laid more eggs in the presence of odors from these bacteria (Lance, 1992).

Virtually no studies have explored how rootworm activity affects the bacterial flora associated with soil and maize roots, although impacts of root herbivory by other invertebrates have been investigated. Infestation with plant-parasitic nematodes can alter the rhizobacterial flora of tomato (O'Flaherty et al., 2003), enhance soil nutrient cycling by microbes (Tu et al., 2003), increase the soil microbial biomass associated with white clover roots (Denton et al., 1999), or have no effect on the latter parameter (Ayres et al., 2007). Furthermore, microbe substrate utilization patterns were altered when rhizosphere solutions from nematode-damaged white clover roots were added to soil, although there was no change in the bacterial community structure (Treonis et al., 2007). The soil microbial community associated with grassland plants can be altered by larval cranefly herbivory (Grayston et al., 2001; Dawson et al., 2004), or by adding rhizosphere solutions from damaged roots to the soil (Treonis et al., 2005), while damage to maize roots by rootworms does not affect soil microbial biomass or respiration under greenhouse conditions (Treonis and Zasada, 2005).

In summary, given the consistent occurrence of *S. grimesii* on maize roots and its role as a fungal antagonist in other rhizosphere systems (Lottmann et al., 1999, 2000; Berg et al., 2002), we believe this species may have a similar function in the maize rhizosphere, and has potential as a plant growth promotion candidate (Compant et al., 2005). With regard to *S. marcescens* A1b, it is likely that *Serratia*-infested rootworm larvae introduced these microbes into the maize rhizosphere, although plant-mediated changes may have encouraged the growth of the pink strain. More experiments are needed to answer these questions and determine the effects of isolated bacterial strains on maize growth and rootworm life-history parameters. Consistent association of the *S. marcescens* A1b orange strain with diseased rootworms indicates that it may have potential as a biocontrol agent in a similar manner as the *Serratia* spp. associated with amber disease in grass grubs (Jackson et al., 2001). The repeated association of *S. marcescens*, *P. aeruginosa* and *K. pneumoniae* in diseased adult rootworms suggests a reservoir for opportunistic pathogens in the environment that may extend beyond the plant rhizosphere described by Berg et al. (2005b).

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